

Interleukin-6 Reduces Cartilage Destruction during Experimental Arthritis

A Study in Interleukin-6-Deficient Mice

Fons A. J. van de Loo,* Sandra Kuiper,*
Frank H. J. van Enkevort,[†] Onno J. Arntz,*
and Wim B. van den Berg*

From the Departments of Rheumatology* and
Endocrinology,[†] University Hospital Nijmegen, Nijmegen,
The Netherlands

Using interleukin (IL)-6-deficient (IL-6^{0/0}) mice or wild-type mice, we investigated the controversial role of IL-6 in joint inflammation and cartilage pathology during zymosan-induced arthritis (ZIA). Monoarticular arthritis was elicited by injection of zymosan into the right knee joint cavity. Production of IL-1, tumor necrosis factor (TNF), IL-6, and nitric oxide by the inflamed knee was assessed in washouts of joint capsule specimens. Plasma corticosterone was measured using a radioimmunoassay. Proteoglycan synthesis was assessed using [³⁵S]sulfate incorporation into patellas ex vivo. Joint swelling was quantified by joint uptake of circulating ^{99m}Tc-hexamethylenetriamine technetium pertechnetate. Histology was taken to evaluate cellular infiltration and cartilage damage. Zymosan caused a rapid increase in articular IL-1, IL-6, TNF, and NO levels. Except for IL-6, the released amounts and time course of these mediators were comparable in the IL-6-deficient mice and the wild-type mice. Elevated plasma corticosterone levels were measured during the first day of arthritis in both strains. At day 2 of ZIA, joint inflammation (joint swelling and cell exudate) in IL-6-deficient mice was comparable with that in the wild-type mice. The marked suppression of chondrocyte proteoglycan synthesis and proteoglycan degradation were on the average higher in the IL-6-deficient mice. Together this resulted in a more pronounced proteoglycan depletion in the IL-6-deficient mice as compared with the wild-type mice during the first week of arthritis. Injection of recombinant IL-6 into the

joint cavity corrected the IL-6 deficiency and significantly reduced cartilage destruction. Inflammation was more chronic in the wild-type mice, and these mice also showed a higher prevalence for osteophyte formation. In ZIA, IL-6 plays a dual role in connective tissue pathology, reducing proteoglycan loss in the acute phase and enhancing osteophyte formation in the chronic phase. The latter could be related to the more severe joint inflammation as seen in the normal (IL-6-producing) animals during the chronic phase of arthritis. (Am J Pathol 1997, 151:177–191)

Interleukin (IL)-6 is a glycosylated polypeptide of approximately 26 kd made by lymphoid and nonlymphoid cells. It belongs to a family of proteins with overlapping functions: leukemia inhibitory factor, oncostatin M, ciliary neurotrophic factor, and IL-11. The IL-6 receptor consists of two subunits, the α -chain, with the IL-6 binding site, and the signal-transducing β -chain (gp130). The gp130 receptor is shared by all members of the IL-6 family, which may explain why they have many activities in common.^{1–3}

IL-6 is present in large quantities in synovial lavage of patients with arthritic disorders.^{4–7} In rheumatoid arthritis (RA), synovial fluid IL-6 exceeded the plasma IL-6 levels in these patients.⁸ Synovial fibroblasts and the articular chondrocytes are identified as the main IL-6 producers in the inflamed joint.^{9–13} IL-6 is a pleiotropic mediator and is thought to be involved in both systemic and local events in the arthritic patient. IL-6 plays an important role in host defense, regulating cellular and humoral immune re-

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Address reprint requests to Dr. Fons van de Loo, Department of Rheumatology, University Hospital Nijmegen, Geert Grooteplein Zuid 8, 6525 GA Nijmegen, The Netherlands.

sponses, in particular, B cell maturation into plasma cells.^{14–16} Furthermore, IL-6 is a potent inducer of acute-phase protein synthesis in hepatocytes,¹⁷ and increased serum IL-6 levels correlated with an enhanced acute-phase protein response in RA patients.^{1,8,18} At the local level, IL-6 promotes differentiation of macrophages and is an accessory protein in T cell activation and antibody secretion,^{1–3,19,20} which suggests that IL-6 is at least involved in some features of the joint pathology. On the other hand, IL-6 can induce IL-1 receptor antagonist, soluble tumor necrosis factor (TNF) receptors and tissue inhibitor of metalloproteinases (TIMP) in articular cells^{21,22} and reduce TNF production,²³ which suggests that IL-6 may reduce joint pathology. Some studies reported that IL-6 levels correlated with disease activity,^{4,10} whereas others reported that the increase in serum IL-6 levels coincided with clinical improvements.²⁴ Furthermore, a recent study showed that there was no correlation with radiological progression of joint damage.¹⁸ There is, however, no direct evidence that IL-6 may play a role in the pathogenesis of RA. Studies in animal models may shed light on the ambiguous role of IL-6 in arthritis.

Recently we performed a study using IL-6 neutralizing antibodies in antigen-induced arthritis and failed to demonstrate a role for IL-6 in inflammation and cartilage destruction in this model.²⁵ Proteins of approximately 60 kd are unable to penetrate the cartilage matrix,²⁶ making it unlikely that chondrocyte-derived IL-6 was blocked by the 150-kd large antibodies. For this, we couldn't conclude that IL-6 was not involved in the observed cartilage destruction. Recently, several groups developed mice with a disrupted IL-6 gene by homologous gene recombination to study the *in vivo* role of IL-6 in bacterial and viral infection, fever response, and bone resorption.^{27–34} We chose to study the effect of IL-6 deficiency on the local events of arthritis, ie, joint inflammation and cartilage destruction. It is clear that these mice exhibit an impaired immune response.²⁷ For this, the zymosan-induced arthritis (ZIA) model was chosen, as the inflammation was not immunologically mediated.³⁵ It was found that the onset was comparable in the IL-6-deficient mice, their wild-type controls (C57Bl/6 \times 129Sv), or the C57Bl/6 mice. We clearly demonstrated that, in the arthritic IL-6-deficient mice, cartilage destruction was more pronounced, whereas fewer animals developed osteophytes as compared with the wild-type mice.

Materials and Methods

Animals

Homozygous IL-6^{o/o} and wild-type (C57Bl/6 \times 129/Sv)F2 mice were obtained from M. Kopf (Basel, Switzerland) and bred in our own animal facilities, as were the C57Bl/6 mice. Mice were housed in filter-top cages under standard pathogen-free conditions and fed a standard diet and acidified tap water *ad libitum*. At the age of 8 to 10 weeks they were used in the experiments.

Recombinant Cytokines and Antibodies

Purified and biologically active mature murine recombinant IL-1 α , cloned in *Escherichia coli*, was generously donated by I. G. Otterness (Pfizer Central Research, Groton, CT), and bioactivity was checked in a bioassay. Purified murine recombinant IL-6 was a gift from G. Ciliberto (I.R.B.M., Rome, Italy).

Control of IL-6 Deficiency

The IL-6 gene was disrupted in the second exon by insertion of a *neo* cassette.²⁷ Loss of wild-type IL-6 messenger RNA was confirmed by reverse transcriptase polymerase chain reaction (RT-PCR) using primers bridging the insertion: 5' primer 5'TCT GCA AGA GAC TTC CAT CCA 3' and 3' primer 5'GCA AGT GCA TCA TCG TTG TTC 3',³³ purchased from Pharmacia Biotech (Roosendaal, The Netherlands). Controls were included for a possible bypass of IL-6 by IL-11. Sequences of the primers for IL-11 were upstream (5', 5'CTG TGG GGA CAT GAA CTG TG 3') and downstream (3', 5'AGC CTT GTC AGC ACA CCA G 3'). Messenger RNA from cartilage or synovial tissue, isolated with TRIzol reagent according to the protocol of the manufacturer (Life Technology, Breda, The Netherlands), was reverse transcribed to cDNA using oligo-dT primers by standard protocol. One-twentieth of the cDNA was used for one PCR reaction of 35 cycles: denaturation at 92°C for 1 minute and annealing at 55°C for 1 minute, followed by elongation with Taq DNA polymerase (Life Technologies) at 72°C for 1 minute. The expected PCR products of IL-6 and IL-11 were 239 and 300 bp, respectively. This control was performed regularly during breeding of the animals. In cartilage obtained from IL-6^{o/o} mice, no IL-6 mRNA was detected, whereas IL-6 mRNA was present in cartilage from IL-6^{+/+} mice, and marked enhancement was found after IL-1 challenge. There was no evidence of enhanced IL-11 expression in cartilage from IL-6^{o/o}

mice. Second, culture supernatant of articular cartilage was checked for IL-6 bioactivity using the B9 assay, and no bioactive IL-6 was found in conditioned medium of cartilage cultures from IL-6^{o/o} mice.

Zymosan-Induced Arthritis

A homogeneous suspension of 30 mg of zymosan A (*Saccharomyces cerevisiae*), dissolved in 1 ml of endotoxin-free saline, was obtained by boiling twice and sonic emulsification. Arthritis was induced by intra-articular injection of 180 μ g of zymosan along the suprapatellar ligament into the joint cavity. The contralateral knee joint received an equal amount of saline (6 μ l) and served as a within-animal control.

Assessment of Joint Swelling

Animals were injected subcutaneously with 10 μ Ci of ^{99m}Tc-pertechnetate (^{99m}Tc) in 0.2 ml of saline in the neck region. After 15 minutes, mice were sedated by intraperitoneal injection of 4.5% chloral hydrate, 0.1 ml/10 mg of body weight. The accumulation of the isotope due to the increased blood flow and edema in the knee was determined by external gamma counting and expressed as the ratio of the ^{99m}Tc uptake in inflamed over contralateral knee joint. A ratio higher than 1.1 indicates joint swelling.

Assessment of Cytokine Production by Arthritic Joints

Patellas were dissected with surrounding soft tissue, consisting of the tendon and synovium, in a standardized manner. Each patella was incubated in 200 μ l of serum-free RPMI 1640 for 1 hour at 37°C; thereafter, medium was changed and incubated for another 24 hours. Both 1- and 24-hour culture supernatants were stored at -70°C preceding cytokine and nitrite measurements, respectively.

Assessment of Cytokines in 1-Hour Washouts of Knee Joint Capsule Specimens

IL-1 activity was measured in the one-stage proliferation assay as described by Gearing et al.³⁶ The murine thymoma cell line EL-4 NOB-1 (ECACC, Porton Down, Salisbury, UK) was used as an IL-1-specific cell producing IL-2 in response, in combination with the IL-2-sensitive CTLL2 cells (ECACC). The

cells were plated out in concentrations of 1×10^5 /well NOB1 cells and 4×10^3 /well CTLL cells in RPMI supplemented with 5% fetal calf serum for 21 hours.

IL-6 activity was determined by a proliferative assay using B9 cells. Briefly, 5×10^3 B9 cells in 200 μ l of 5% fetal calf serum/RPMI 1640 per well were plated in a round-bottom microtiter plate and incubated for 3 days using human recombinant IL-6 as standards. At the end of the incubation (both IL-1 and IL-6 assay), 0.5 μ Ci of [³H]thymidine (specific activity, 20 Ci/mmol; Dupont NEN, Boston, MA) was added per well. Three hours later, cells were harvested and thymidine incorporation (NOB1 cells are thymidine kinase deficient) was determined. The detection limit of the IL-1 assay was 0.1 pg/ml murine recombinant IL-1 and for the IL-6 assay was 1 pg/ml.

TNF in culture supernatants was measured in duplicate by non-equilibrium radioimmunoassay (RIA) as described elsewhere.³⁷ In short, standards and culture supernatants were diluted in a RIA buffer containing 60 mmol/L Na₂HPO₄·2H₂O, 12 mmol/L sodium EDTA; 0.02% sodium azide, 0.25% bovine serum albumin (RIA grade, Sigma Chemical Co., St. Louis, MO), 0.1% Triton X-100, and 250,000 kallikrein-inactivating units of aprotinin (Bayer, Leverkusen, Germany), pH 7.4. A 100- μ l volume of an appropriate rabbit anti-TNF antiserum dilution in RIA buffer was added to 100 μ l of samples and standards and kept on ice. After vortexing, the tubes were incubated for 24 hours at 4°C. Subsequently, 100 μ l of the appropriate ¹²⁵I-labeled TNF- α containing approximately 10,000 cpm was added to each tube, and incubation was continued for another 24 hours at 4°C. To separate bound and free tracer, 750 μ l of RIA buffer containing 9% (w/v) polyethylene glycol 6000 (Merck Diagnostica, Darmstadt, Germany) and 3% (w/v) goat anti-rabbit serum were added. The tubes were incubated for 20 minutes at room temperature and then centrifuged at $1500 \times g$ for 15 minutes. Supernatants were discarded carefully and quickly drained on absorbent paper. Remaining radioactivity was counted in a gamma counter. The radioactivity in control tubes (the non-specific binding activity) was subtracted from samples and standards.

Nitrite Measurements in 24-Hour Washouts of Knee Joint Capsule Specimens

The medium concentration of NO₂⁻ (a stable breakdown product of NO) was determined by Griess reaction using NaNO₂ standards. The Griess reagent consisted of 0.1% naphthylethylene diamine

dihydrochloride, 1:1 diluted with 1.0% sulfanilamide in 5% H_3PO_4 . Briefly, 100 μl of conditioned medium was mixed with 100 μl of Griess reagent in a flat-bottom microtiter plate and absorbance read at 545 nm using an ELISA plate reader.

Blood Sampling and Corticosterone Measurements

Mice were anesthetized by ether inhalation, and blood samples were collected within 5 minutes in dry lithium/heparin glass tubes and centrifuged at $1500 \times g$ for 10 minutes at 4°C . Plasma was separated and stored at -20°C until assayed. Corticosterone was measured by RIA as described by Sweep et al.³⁸ Briefly, plasma corticosterone was extracted with 7.5 ml of dichloromethane (Baker, Deventer, The Netherlands). The water phase was discarded and the dichloromethane phase was evaporated. The residue was dissolved in 2 ml of 0.2% ethylene glycol/water, and the concentration of corticosterone in the eluate was measured by RIA using an antiserum raised in sheep against a B-21-hemisuccinate-bovine serum albumin conjugate. Corticosterone tracer ($[1\alpha,2\alpha\text{-}^3\text{H}]$ corticosterone; Amersham, Little Chalfont, UK) was used in the extraction and in the RIA procedure. The sensitivity of the assay was 25 to 45 fmol/tube. The intra- and interassay coefficients of variation were 10 and 16.7%, respectively.

Assessment of Chondrocyte Proteoglycan Synthesis

Patellas were dissected with a minimal amount of adjacent soft tissue and placed in RPMI 1640 supplemented with gentamicin (50 mg/L), L-glutamine (2 mmol/L), and 40 μCi of $[^{35}\text{S}]$ sulfate. At the end of the 3-hour incubation period, nonincorporated free radiolabel was removed by thorough washing of the patellas in saline (three times for 10 minutes each) and fixed overnight in 10% formalin. After decalcification in formic acid (5%) for 4 hours, the whole patellas could easily be punched out (agar gel punch of 1 mm diam.). The patellas were dissolved in 0.5 ml of Lumasolve (Lumac, Groningen, The Netherlands) overnight at 60°C . The ^{35}S content of each patella was measured by liquid scintillation counting and expressed as counts per minute (cpm). More than 95% of the radiolabel was incorporated into the glycosaminoglycans of proteoglycans.^{39,40} Data are represented as total incorporation of $[^{35}\text{S}]$ sulfate or as a ratio of right over left knee (within-animal control value) as paired values.

Assessment of Cartilage Proteoglycan Degradation

Radioactive sulfate (50 μCi of $[^{35}\text{S}]$ sulfate/200 μl of saline) was injected into the peritoneal cavity 1 day before arthritis induction.⁴⁰ At day 2 of arthritis, patellas were dissected and processed for liquid scintillation counting as described in the previous section.

Histological Processing and Analysis of Knee Joints

Knee joints were dissected, fixed, decalcified, dehydrated, and embedded in paraffin. Standard frontal sections of 7 μm were prepared and stained with safranin-O and counterstained with fast green. Serial sections of the whole knee joints were scored by two independent blinded observers. Cartilage depletion was scored using a linear scale from 0 (normal safranin-O staining, indicating no depletion) up to 3 (complete loss of safranin-O staining, indicating full depletion). Furthermore, the presence of chondrophytes (cartilaginous tissue) and osteophytes (ossified tissue) adjacent to the patellas was evaluated histologically and the incidence was determined in the different mouse strains.

Statistical Analysis

Data are expressed as mean values \pm SD unless stated otherwise. Statistical significance was tested using the Wilcoxon's rank sum test or with the Student's *t*-test as stated.

Results

Zymosan-Induced Cytokine Release in Murine Knee Joints

An intra-articular injection of zymosan triggers the release of the pro-inflammatory cytokines IL-1, TNF- α , and IL-6 by the synovial joint capsule (Figure 1, A-C). No IL-1 and IL-6 was present in washouts of tissues taken from normal joints. In contrast, considerable amounts of TNF were found in these control supernatants, although it was not biologically active. The largest amounts of IL-1 and IL-6 were found in washouts of tissues from joints taken 3 and 6 hours after zymosan injection, respectively. At these time points, higher amounts of immunoreactive TNF were also measured in these washouts. Although IL-6 levels exceeded those of IL-1 and TNF at the molar

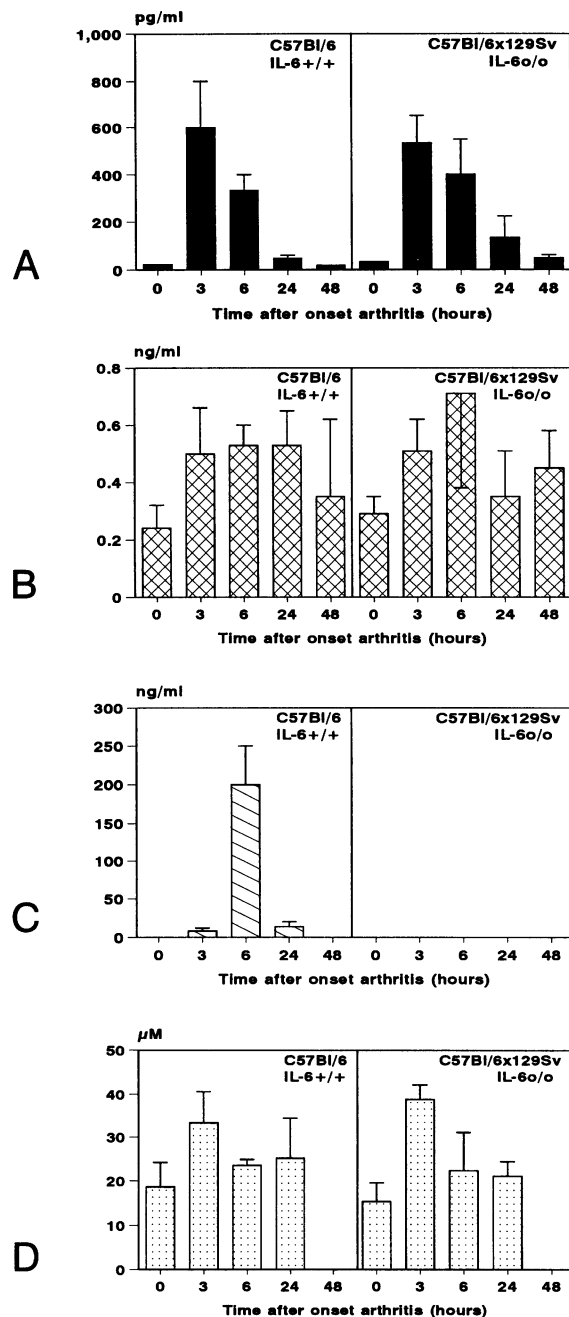


Figure 1. IL-1, TNF, IL-6, and NO release ex vivo by specimen of the articular synovial capsule dissected during zymosan-induced arthritis. Patellas with adjacent soft tissue were dissected in a standardized manner to obtain large specimens of synovia. Patellas were incubated in 200 μ l of RPMI for 1 hour at room temperature followed by 24 hours at 37°C for assessment of IL-1 by NOB/CTLL proliferation assay (A), TNF by RIA (B), IL-6 by B9 proliferation assay (C), and NO by Griess reagent (D), as described in Materials and Methods. Data represent the mean value of four patellas per time point. IL-1 and NO levels between IL-6^{+/+} and IL-6^{o/o} mice were not significantly different. IL-6 in conditioned media of patellas from IL-6^{o/o} mice could not be detected using the B9 assay.

base, IL-1 and TNF release was IL-6 independent as became evident from zymosan injections into IL-6-deficient mice (Figure 1).

Increased Nitric Oxide Release after Zymosan Injection

Increased nitrite levels were measured in the 24-hour culture supernatants of synovial joint capsule specimens taken 3, 6, and 24 hours after zymosan injection (Figure 1D). Levels were not significantly different between the IL-6-deficient mice and the wild-type C57Bl/6 mice, suggesting that IL-6 also was not involved in NO production in zymosan-injected knees.

Plasma Corticosterone and IL-6 Levels during Zymosan-Induced Arthritis

Increased plasma corticosterone levels were measured at 3 and 6 hours after induction of arthritis in the wild-type mice (Table 1). Corticosterone values returned to baseline within 24 hours after zymosan injection. Plasma IL-6 levels were also raised 3 and 6 hours after zymosan injection intra-articularly and returned to baseline at 48 hours in the wild-type mice. By comparison, the plasma IL-6 levels in the wild-type mice were far below the amounts found in the washouts of inflamed synovial joint capsule specimens of these animals, but both reached higher values at 6 hours and already markedly declined at 24 hours after arthritis induction (Table 1; Figure 1C).

Time course and plasma levels of corticosterone in the IL-6-deficient mice were indistinguishable from the response in the wild-type mice (Table 1). The corticosterone response seemed to be IL-6-independently regulated as no IL-6 was detected in these plasma samples (Table 1).

Zymosan-Induced Joint Inflammation

Enhanced blood flow and plasma extravasation (edema) resulted in an increased uptake of circulating ^{99m}Tc isotope by the inflamed knee joint. Joint swelling at day 2 of ZIA was not different between the IL-6-deficient mice, the wild-type mice, and the C57Bl/6 strain (Table 2). At day 4 of ZIA, joint swelling had subsided significantly more in the IL-6-deficient mice as compared with the IL-6^{+/+} strains (Table 2).

Enhanced Cartilage Destruction in IL-6-Deficient Arthritic Mice

Marked inhibition of chondrocyte proteoglycan synthesis and accelerated proteoglycan breakdown

Table 1. Plasma Corticosterone and IL-6 Levels during Arthritis

Time of arthritis (hours)	C57Bl/6 (IL-6 ^{+/+}) mice		C57Bl/6 × 129Sv (IL-6 ^{0/0})	
	Corticosterone (nmol/L)	IL-6 (ng/ml)	Corticosterone (nmol/L)	IL-6 (ng/ml)
0	123 ± 86	0.23 ± 0.06	266 ± 62	0.13 ± 0.00*
3	894 ± 152	2.56 ± 1.02	789 ± 77	0.22 ± 0.06
6	741 ± 295	6.14 ± 2.36	651 ± 196	0.22 ± 0.06
24	282 ± 54	1.02 ± 0.72	166 ± 41	0.16 ± 0.08
48	161 ± 101	0.32 ± 0.13	159 ± 177	0.13 ± 0.00

Corticosterone was measured by RIA as described in Materials and Methods. Plasma corticosterone levels in untreated mice ranged between 20 and 400 nmol/L. The values at 3 and 6 hours were not significantly different between normal and the IL-6-deficient mice. IL-6 was measured using a bioassay as described in Materials and Methods. The number of animals per time point evaluated was four per mouse strain.

*Detection limit for nonspecific stimulation of B9 cells using plasma.

was measured in patellas at day 2 of arthritis (Table 3). Both inhibition of proteoglycan synthesis and breakdown were moderately but significantly higher in the IL-6-deficient mice. The combined effect was a more pronounced loss of cartilage glycosaminoglycans in the IL-6-deficient mice ($-28 \pm 10\%$) as compared with the wild-type mice ($-7 \pm 9\%$) at day 2 of arthritis.

IL-1-Induced Proteoglycan Synthesis Suppression and Degradation

We further evaluated the chondrocyte responsiveness to IL-1 in normal and IL-6-deficient mice. Murine recombinant IL-1 α was injected intra-articularly, and patellar proteoglycan synthesis and degradation were measured 24 hours later. IL-1 caused a distinct inhibition of synthesis and only a moderate breakdown of proteoglycans (Table 4). In the IL-6-deficient mice, IL-1-induced suppression and deg-

radation were not different, suggesting that the chondrocytes in these mice were not more vulnerable for IL-1 and that IL-6 has no major role in these processes.

Role of IL-6 in Joint Pathology during Zymosan-Induced Arthritis

Histological sections of whole knee joints showed severe inflammation at days 2, 4, and 7 after zymosan injection. At day 2, plasma exudation was a prominent feature (Table 2), and large numbers of polymorphonuclear neutrophils entered the joint space (Table 5; Figure 2). At day 7 of arthritis, synovitis became the prominent inflammatory feature in the wild-type mice and synovitis was less severe in the IL-6-deficient mice (Table 5). Cartilage proteoglycan depletion was already evident at day 2 of inflammation and progressed into severe loss at day 7 of inflammation (Table 5; Figure 2). At day 2 of the

Table 2. Joint Swelling in Zymosan-Induced Arthritis

Time (day)	^{99m} Tc uptake (R/L ratio)		
	C57Bl/6 mice (IL-6 ^{+/+})	C57Bl/6 × 129Sv (IL-6 ^{0/0})	C57Bl/6 × 129Sv (IL-6 ^{+/+})
2	1.57 ± 0.09 (13)	1.53 ± 0.20 (18)	1.56 ± 0.17 (14)
4	1.18 ± 0.09 (13)*	1.06 ± 0.10 (13)	1.15 ± 0.09 (13)†

Joint swelling is expressed as a ratio of ^{99m}Tc uptake by the arthritic joint (R) over the normal contralateral knee joint (L). Joint swelling was significantly different from the C57Bl/6 × 129Sv (IL-6^{0/0}) mice: * $P < 0.01$; † $P < 0.05$ (Student's *t*-test). The number of animals per group is indicated in parentheses.

Table 3. Proteoglycan Turnover during Arthritis in Normal and IL-6-Deficient Mice

Mouse strain	Proteoglycan synthesis		Proteoglycan breakdown	
	Ratio (cpm _R /cpm _L)	Inhibition (%)	Ratio (cpm _R /cpm _L)	Enhanced loss (%)
C57Bl/6 (IL-6 ^{+/+})	0.42 ± 0.11* (9)	58	0.66 ± 0.07* (16)	34
C57Bl/6 × 129Sv (IL-6 ^{0/0})	0.33 ± 0.07 (9)	67	0.57 ± 0.09 (14)	43
C57Bl/6 × 129Sv (IL-6 ^{+/+})	0.42 ± 0.19 (6)	58	0.64 ± 0.08* (16)	36

Proteoglycan synthesis and proteoglycan degradation were measured at day 2 of ZIA as described in Materials and Methods and expressed as the ratio of [³⁵S]sulfate content (cpm) in the arthritic knee joint (R) over the normal contralateral joint (L). Number of animals per group is indicated in parentheses.

*Values are significantly different between IL-6-deficient (0/0) and the IL-6-producing (+/+) mouse strains: $P < 0.05$ (Student's *t*-test).

Table 4. Effect of IL-1 Injection on Proteoglycan Turnover in IL-6-Deficient Mice and IL-6^{+/+} Mice

Mouse strain	Proteoglycan synthesis		Proteoglycan breakdown	
	Ratio (cpm _H /cpm _L)	Inhibition (%)	Ratio (cpm _H /cpm _L)	Enhanced loss (%)
C57Bl/6 (IL-6 ^{+/+})	0.59 ± 0.07 (7)	41	0.91 ± 0.13 (7)	9
C57Bl/6 × 129Sv (IL-6 ^{0/0})	0.59 ± 0.13 (7)	41	0.89 ± 0.08 (7)	11

Proteoglycan synthesis was measured at day 1 after injection, and proteoglycan breakdown was measured at day 2 after injection of 10 ng of murine recombinant IL-1 α . Expression of proteoglycan synthesis is as explained in Table 3. Number of animals per group is indicated in parentheses.

joint inflammation, cartilage proteoglycan loss was significantly higher in the IL-6-deficient mice as compared with the IL-6^{+/+} mice strains. This difference in proteoglycan loss was still present at day 7 of arthritis, although the ongoing inflammation became more severe in the wild-type mice (Table 5).

Effect of IL-6 Administration to IL-6-Deficient Mice on Zymosan-Induced Arthritis

To prove the ameliorating role of IL-6 on cartilage destruction during arthritis, knockout mice received recombinant IL-6 locally. Recombinant IL-6, either applied as a co-injection with zymosan or injected 3 hours after the zymosan injection, reversed the enhanced cartilage destruction in these knockout mice (Table 6). IL-6 supplementation did not suppress joint inflammation, and this again proves that the onset of inflammation was IL-6 independent.

Osteophyte Formation in Zymosan-Induced Arthritis

The first signs of osteophyte formation (chondrocytes) were seen at day 7 of arthritis, adjacent to the patellas (Table 7). Of 24 normal mice, 13 devel-

oped significant osteophytes, compared with only 2 of 26 IL-6-deficient mice. At day 14, the incidence was 6 of 6 mice in the normal group and 3 of 6 mice in the IL-6-deficient group. The total number of osteophytes developed at both lateral and medial sites of the patella (Figure 2) was also higher in the IL-6^{+/+} group as compared with the IL-6-deficient group (Table 7).

Discussion

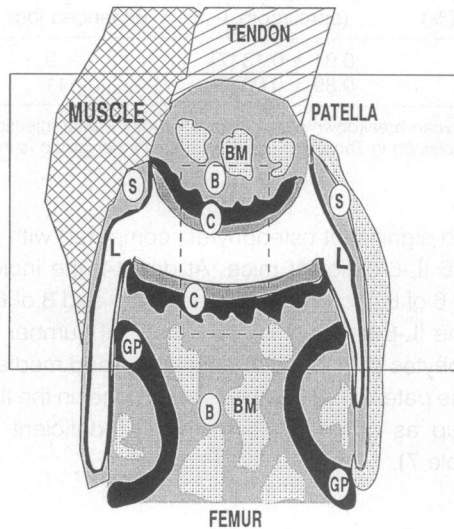
Using IL-6-deficient (IL-6^{0/0}) mice or wild-type mice, we provided direct evidence that IL-6 was involved in cartilage pathology in an experimental model of arthritis. Cartilage proteoglycan depletion in the early phase of ZIA was significantly higher in the IL-6-deficient mice, whereas joint inflammation was comparable between the strains, suggesting that IL-6 ameliorates cartilage proteoglycan destruction. Furthermore, IL-6-deficient mice developed fewer number of osteophytes in the chronic phase of arthritis. This is probably related to the significantly milder joint swelling and synovitis from day 4 of arthritis in the IL-6-deficient mice as compared with the wild-type mice.



Table 5. Histological Evaluation of Zymosan-Induced Arthritis in Normal and IL-6-Deficient Mice

Group	Cellular infiltrate	Proteoglycan depletion	
		Patellar cartilage	Femoral cartilage
Day 2 ZIA			
C57Bl/6 (IL-6 ^{+/+})	2.23 ± 0.86 (40)	0.65 ± 0.46* (40)	1.05 ± 0.69* (49)
C57Bl/6 × 129Sv (IL-6 ^{0/0})	2.28 ± 0.99 (40)	1.61 ± 0.73 (40)	1.90 ± 0.85 (46)
C57Bl/6 × 129Sv (IL-6 ^{+/+})	2.32 ± 0.95 (19)	0.92 ± 0.48* (19)	1.30 ± 0.71* (25)
Day 4 ZIA			
C57Bl/6 (IL-6 ^{+/+})	1.21 ± 0.27 (7)	1.50 ± 0.71* (7)	1.79 ± 0.64* (7)
C57Bl/6 × 129Sv (IL-6 ^{0/0})	0.93 ± 0.35 (7)	2.29 ± 0.39 (7)	2.43 ± 0.45 (7)
C57Bl/6 × 129Sv (IL-6 ^{+/+})	1.00 ± 0.50 (7)	1.25 ± 0.55* (7)	1.43 ± 0.61* (7)
Day 7 ZIA			
C57Bl/6 × 129Sv (IL-6 ^{0/0})	1.44 ± 0.85 (26)	2.08 ± 1.03 (26)	2.15 ± 1.08 (26)
C57Bl/6 × 129Sv (IL-6 ^{+/+})	1.90 ± 0.90 (24)	1.52 ± 1.05 ^{ns} (24)	1.63 ± 0.98 ^{ns} (24)

Serial histological sections of knee joints were scored by blinded observer. For joint inflammation, at day 2 and 4, the extent of exudated neutrophils was scored, and at day 7, synovitis was scored. Cartilage depletion was scored as loss of safranin-O staining. A maximal score of 3 indicates no staining of the superficial cartilage layers (see Figure 2C). Cartilage depletion was statistically tested between IL-6-deficient (0/0) and the IL-6-producing (+/+) mouse strains: **P* < 0.01 (significantly different using the Wilcoxon rank sum test). ns, not significant. Number of animals per group is indicated in parentheses.

A Cross-section knee joint



B, Bone; BM, Bone Marrow;
 C, Cartilage; GP, Growth Plate;
 L, Lining; S, Synovium. Photograph 
 higher magnification insert 

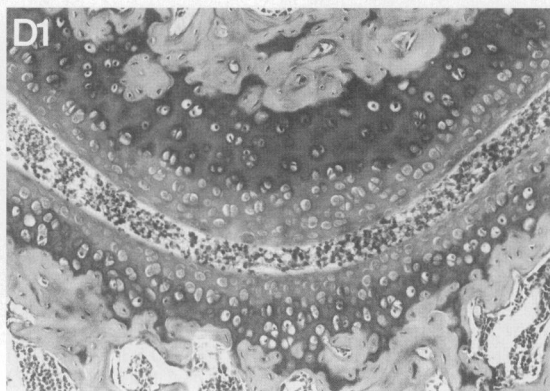
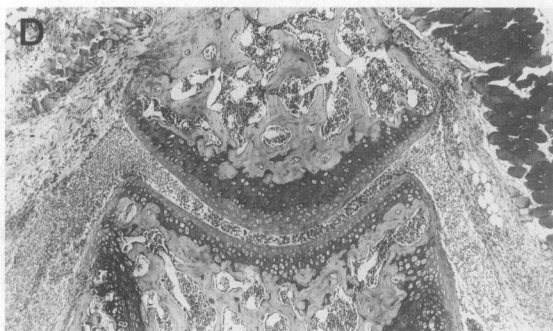
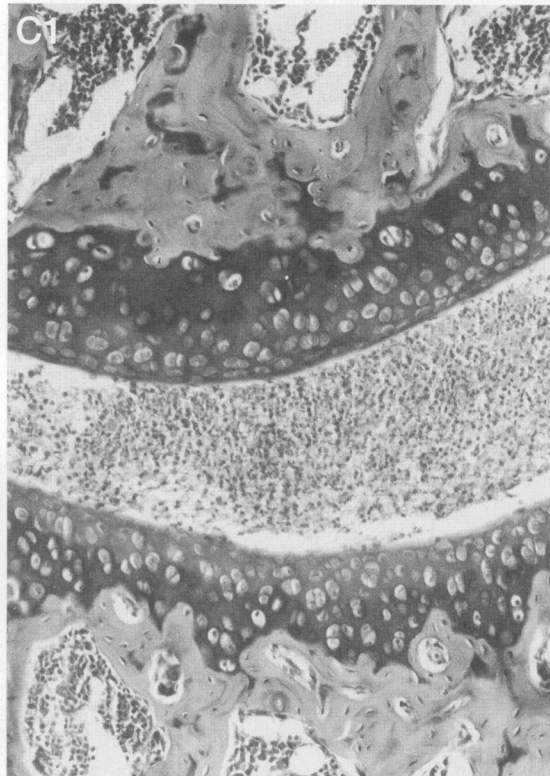


Figure 2. Histological section of normal knee joints (B and K) and knee joints injected with zymosan (C to J). Serial sections were stained with safranin-O and scored in a blinded fashion. A: Schematic representation of the femoropatellar region of a knee joint section, indicating the different articular tissues and the site taken for the higher magnification insets (C1 and D1). Safranin-O staining was lost in the superficial cartilage layers in the femoropatellar area at day 2 of ZIA in the C57BL/6 mouse (C, C1) and was more pronounced in the IL-6-deficient mouse (D, D1). This was also seen in the femorotibial area (E, wild type; F, IL-6-deficient mice). Note that the presence of neutrophils in the joint cavity and synovium was similar in both the wild-type and IL-6-deficient mouse. At day 7 of ZIA, cartilage proteoglycan depletion was more pronounced (G) and still higher in the IL-6-deficient mice (H). Osteophytes were present at day 14 of ZIA in the C57BL/6 mouse (I) as indicated by the arrowheads, and reduced numbers were seen in the IL-6-deficient mouse (J). No osteophytes were seen in age-matched IL-6-deficient (K) or wild-type mice. Magnification, $\times 100$.

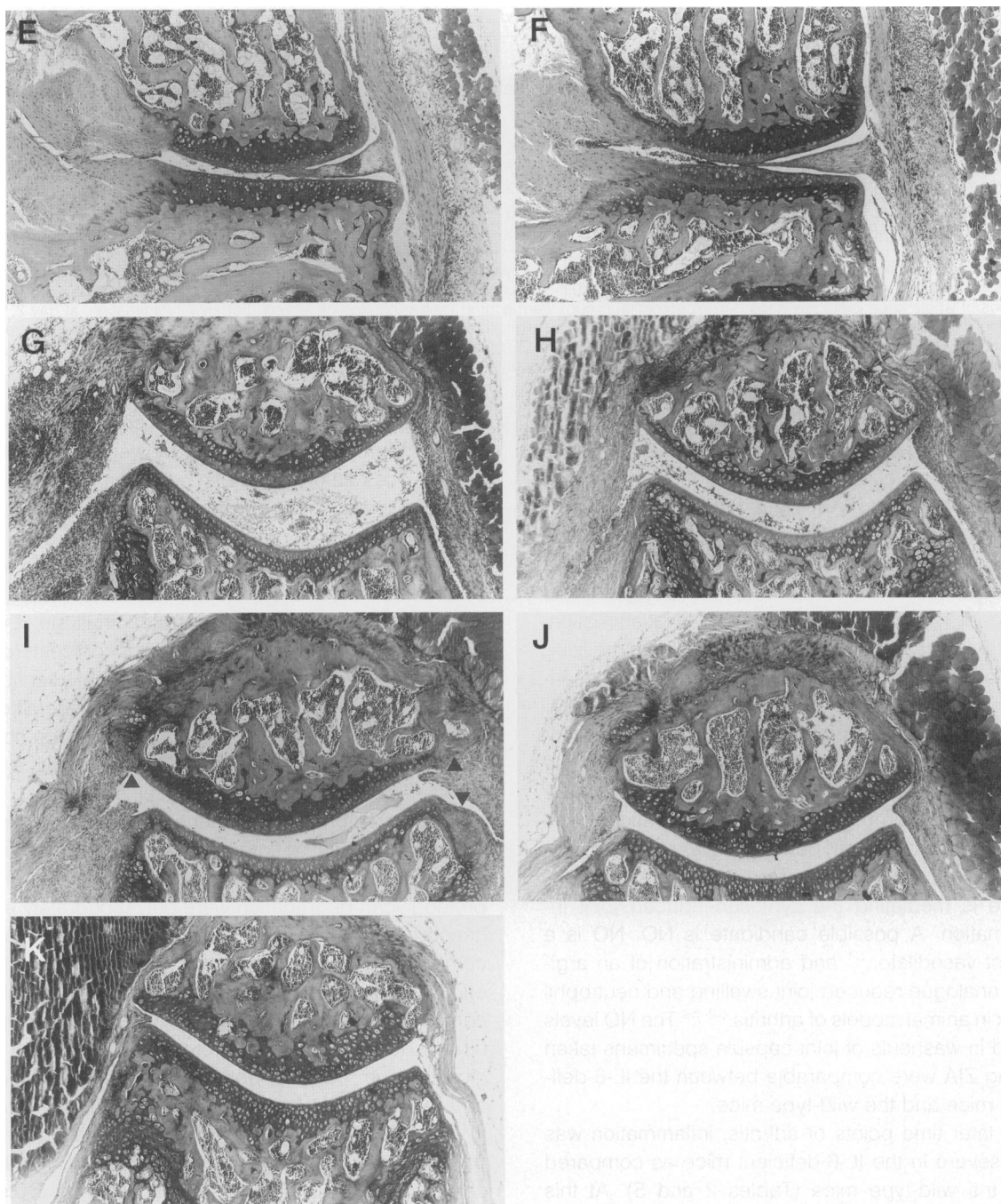


Figure 2.

Opsonized zymosan is a potent trigger for IL-1, TNF, and IL-6 synthesis in murine macrophages *in vitro* and *in vivo*.⁴¹⁻⁴³ Intra-articular injection of zymosan also triggered the release of these pro-inflammatory mediators locally. Comparable amounts of bioactive IL-1 and immunoreactive TNF were found in washouts of joint capsule specimens taken from IL-6-deficient mice and wild-type mice. This suggests that IL-6 was not involved in inducing either

IL-1 or TNF synthesis. At least for IL-1, the balance of cytokine/cytokine inhibitors (IL-1Ra, soluble IL-1 receptors) was not altered by IL-6. IL-1 and TNF are potent inducers of nitric oxide, and we previously demonstrated that murine recombinant IL-6 did not induce NO production in murine chondrocytes and synoviocytes.⁴⁴ Peritoneal macrophages obtained from normal or IL-6-deficient mice produced similar amounts of NO after stimulation with lipopolysaccha-

Table 6. *Effect of IL-6 Supplementation on Zymosan-Induced Arthritis in Knockout Mice*

Group	Cellular infiltrate	Cartilage proteoglycan depletion	
		Patella	Femur
Day 2 ZIA			
C57Bl/6 (IL-6 ^{+/+})	1.83 ± 0.26	0.71 ± 0.25	1.25 ± 0.61
C57Bl/6 × 129Sv (IL-6 ^{+/+})	1.75 ± 0.27	1.33 ± 0.68	1.67 ± 0.82
C57Bl/6 × 129Sv (IL-6 ^{0/0})	1.30 ± 0.57	2.10 ± 0.55	2.00 ± 0.61
IL-6 ^{0/0} ; IL-6 i.a. at 0 hour	1.67 ± 0.68	1.17 ± 0.52*	1.58 ± 0.58
IL-6 ^{0/0} ; IL-6 i.a. at 3 hours	1.50 ± 0.45	1.33 ± 0.68*	1.83 ± 0.61

Arthritis was elicited by intra-articular (i.a.) injection of 180 µg of zymosan. For local IL-6 supplementation in the knockout mice, 900 ng of recombinant IL-6 was either co-injected with zymosan (0 hour) or injected intra-articularly 3 hours after zymosan. Serial histological sections of whole knee joints (six mice per experimental group) were scored by two blinded observers. For joint inflammation, at day 2, the extent of exudated neutrophils was scored. Cartilage depletion was scored as loss of safranin-O staining, with a maximal score of 3 indicating no staining of the superficial cartilage layers (see Figure 2C). Values of cartilage depletion were statistically tested between IL-6-deficient (0/0) and the IL-6-supplemented groups: **P* < 0.05 (significantly different using the Wilcoxon rank sum test).

ride and interferon- γ ,²⁷ suggesting that IL-6 was not a necessary mediator/cofactor in NO synthesis. We also found no effect of the IL-6 deficiency on NO production during ZIA (Figure 1D).

At day 2 of arthritis, joint swelling and inflammation was as severe in the IL-6-deficient mice as in the wild-type mice (Tables 4 and 5). This suggests that IL-6 does not play a role at the onset of inflammation. Treatment of mice with IL-1 and TNF neutralizing antibodies had only a modest effect on zymosan-induced inflammation,²⁵ although their role in inflammation was greater in other animal models of arthritis.^{45–49} Also in the zymosan-induced mouse peritonitis, administration of anti-IL-1 β antibodies had no effect on mononuclear cell migration to the inflamed site and only partly (~40%) reduced neutrophil migration.⁵⁰ It remains to be seen which cytokine is mediating the zymosan-induced joint inflammation. A possible candidate is NO. NO is a potent vasodilator,⁵¹ and administration of an arginine analogue reduced joint swelling and neutrophil influx in animal models of arthritis.^{52–54} The NO levels found in washouts of joint capsule specimens taken during ZIA were comparable between the IL-6-deficient mice and the wild-type mice.

At later time points of arthritis, inflammation was less severe in the IL-6-deficient mice as compared with the wild-type mice (Tables 2 and 5). At this phase, synovitis was prominent and exudate was strongly reduced. We found no clear changes in the

time course of cytokine release that could explain the difference in joint inflammation between the mouse strains. The role of IL-6 is also not clear, and several IL-6 functions could be in place. For example, IL-6 prolongs survival of neutrophils⁵⁵ and maturation of macrophages.^{19,20} Furthermore, corticosteroids are potent anti-inflammatory mediators. This could be of importance as it was demonstrated that the IL-6-deficient mice showed an impaired corticosteroid response to TNF.²⁹ We, however, found identical plasma corticosterone levels during ZIA in the different mouse strains (Table 1), indicating that corticosterone was not responsible for the difference in disease remission between the strains.

This study provided evidence for a modulating role of IL-6 in cartilage pathology. Although the suppression of cartilage proteoglycan synthesis and proteoglycan degradation were only moderately enhanced at day 2 of ZIA in the IL-6-deficient mice as compared with wild-type mice (Table 3), the net effect was a more pronounced cartilage proteoglycan depletion (Table 5; Figure 2). In comparison with other species, cartilage proteoglycan turnover is high in mice. For this, both enhanced degradation and reduced proteoglycan synthesis will contribute to this loss. In the IL-6-deficient mice, the normal bone turnover was significantly increased.³² If the cartilage proteoglycan turnover in these mice is also higher, then suppression of synthesis will contribute even more to the proteoglycan loss in these mice

Table 7. *Occurrence of Osteophytes in Zymosan-Induced Arthritis*

Mouse strain	Day of ZIA	Number of osteophytes	Number of animals with osteophytes	Percentage of animals with osteophytes
C57Bl/6 × 129Sv (IL-6 ^{+/+})	7	16	13	54
	14	9	6	100
C57Bl/6 × 129Sv (IL-6 ^{0/0})	7	3	2	8
	14	4	3	50

Osteophytes developed at the lateral and/or the medial site of the patella during ZIA were counted as seen on serial histological sections of whole knee joints.

during arthritis. Circumstantial evidence was presented by cartilage explant cultures that addition of IL-6 reduced spontaneous proteoglycan release,⁵⁶ suggesting that, in the absence of endogenous IL-6, proteoglycan turnover might be higher.

We previously demonstrated the pivotal role of IL-1 but not of TNF in zymosan-induced cartilage pathology and proved that IL-1 was responsible for the suppression of chondrocyte proteoglycan synthesis in this model.²⁵ Although identical IL-1 levels were found during ZIA, chondrocytes from the IL-6-deficient mice might be more susceptible to IL-1. To investigate this, IL-1 was injected intra-articularly in both mouse strains. Substantial suppression of proteoglycan synthesis and degradation of proteoglycan was found, and the mouse strains responded to IL-1 alike. Whether this also holds true for TNF- or NO-mediated destruction remains to be examined. *In vitro* studies showed that IL-6 stimulates expression of TIMP,²² and a prechallenge with IL-6 reduces TNF production²³ and IL-1-induced cartilage destruction.⁵⁶ This argues in favor of a down-regulating role of IL-6 on cartilage pathology. Furthermore, IL-6 played a pivotal role in the acute-phase response in the RA patients,¹⁷ and some of the acute-phase proteins, eg, α 2-macroglobulin and α 1-proteinase inhibitor, are known to block cartilage-destructive enzymes.⁵⁷ In the wild-type mice, plasma IL-6 levels increased the first day of ZIA with an identical time course as found in the inflamed joints (Table 1). Potentially, the enhanced cartilage destruction during arthritis in the IL-6-deficient mice was caused by the impaired acute-phase response as has been described in these mice.^{27,29,34} Interestingly, the articular IL-6 levels exceeded those in plasma in RA patients,⁸ suggesting that the inflamed joint could be the source of circulating IL-6 during the disease.

Using IL-6-deficient mice, we were able to demonstrate the ameliorating role of IL-6 in cartilage proteoglycan destruction during ZIA in this study. To exclude that the results are caused by strain differences, we used both C57Bl/6 mice and wild-type mice and found no significant differences between the controls. Furthermore, most parameters as measured in this study were not significantly different between the knockout mice and control strains. However, the ultimate proof of the modulating role of IL-6 on cartilage pathology is presented by the intra-articular application of IL-6, which ameliorated the cartilage destruction in the IL-6-deficient mice (Table 6).

We do not claim that IL-6 is the only modulating factor operating during ZIA. Oncostatin M, another member of the IL-6 superfamily, also has the poten-

tial to reduce cartilage degradation by stimulating TIMP-1 expression in chondrocytes.⁵⁸ The anti-inflammatory and chondroprotective action of the Th2-cell-related cytokines IL-4 and IL-10 are impressive,⁵⁹⁻⁶¹ although it remains to be seen whether these cytokines are involved in murine ZIA as this model is not immunologically mediated. Of the growth factors, insulin-like growth factor-1 and the bone-morphogenetic proteins (BMPs) not only are constitutively expressed in cartilage and bone but they also promote synthesis of proteoglycans⁶²⁻⁶⁵ and may antagonize chondrocyte inhibition. Our data, however, clearly showed that IL-6 is an important modulating mediator, ameliorating cartilage proteoglycan destruction.

Osteophyte formation is another feature of the pathology observed in ZIA. Reduced numbers of osteophytes were found in the IL-6-deficient mice (Table 7). In general, osteophyte formation is clearly related to the severity of inflammation and the degree of cartilage destruction.⁶⁶⁻⁶⁸ This relationship was lost in the IL-6-deficient mice because those animals had increased cartilage destruction together with reduced joint inflammation. In the wild-type mice, the increased osteophyte formation could be ascribed to the more severe joint inflammation in the chronic phase of arthritis. These studies also suggest that osteophyte formation probably originates from activation of the periosteum (site of enhanced [³⁵S]sulfate incorporation) rather than from bone cells. Although osteoblasts are potent IL-6 producers, they barely respond to IL-6 themselves, and the osteoclasts are stimulated by IL-6 to resorb bone.² Therefore, it is unlikely that IL-6 activation of osteoclasts caused osteophyte formation. However, it remains to be seen whether IL-6 or other, possibly IL-6-induced, mediators activated the periosteum during arthritis.

We previously found that injections of transforming growth factor (TGF)- β 1 into the murine knee joint resulted in marked osteophyte formation.⁶⁹ Moreover, co-injection of TGF- β completely antagonized IL-1-induced suppression of proteoglycan synthesis in murine cartilage.⁷⁰ It is known that IL-6 is a potent inducer of TGF- β isoforms in human articular chondrocyte cultures⁷¹ and in macrophages.^{72,73} Therefore, reduced TGF- β concentrations in the inflamed joints of IL-6-deficient mice may explain the reduced number of osteophytes and the increased suppression of proteoglycan synthesis as compared with the wild-type mice, and this is currently under investigation. BMP-2 is also a potent stimulator of proteoglycan synthesis in murine chondrocytes and can induce pronounced osteophyte formation *in vivo*.⁷⁴

BMP-2, in contrast to TGF- β , could not antagonize the IL-1-induced suppression of chondrocyte proteoglycan synthesis in murine cartilage,⁷⁴ which qualified BMP-2 as an endogenous mediator in arthritis providing osteophyte formation without affecting proteoglycan synthesis suppression. Reduced BMP-2 expression in the arthritic IL-6-deficient mice may also lead to a lower prevalence for osteophyte formation.

Preliminary results showed that three intra-articular injections of 200 ng of human recombinant TGF- β 2 on alternate days caused pronounced osteophyte formation in both IL-6-deficient and wild-type mice 7 days after the last injection (data not shown). This demonstrated that osteophyte formation *per se* was not impaired in the IL-6-deficient mice.

Some of the controversies of IL-6 in arthritis is probably related to the two faces of IL-6. IL-6 is beneficial in the early phase, reducing cartilage proteoglycan loss probably by inducing inhibitors, and IL-6 is detrimental in the chronic phase, mediating osteophyte formation probably by inducing TGF- β .

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